



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01H 5/00, A01N 63/02, C07K 14/24, C12N 1/11, 1/19, 1/21, 5/14, 7/01	A1	(11) International Publication Number: WO 99/03328 (43) International Publication Date: 28 January 1999 (28.01.99)
(21) International Application Number: PCT/AU98/00562 (22) International Filing Date: 17 July 1998 (17.07.98) (30) Priority Data: PO 8088 17 July 1997 (17.07.97) AU (71) Applicant (for all designated States except US): COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). (72) Inventor; and (75) Inventor/Applicant (for US only): EAST, Peter, David [AU/AU]; 3 Quinn Street, O'Connor, ACT 2602 (AU). (74) Agent: F.B. RICE & CO.; 605 Darling Street, Balmain, NSW 2041 (AU).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: TOXIN GENES FROM THE BACTERIA <i>XENORHABDUS NEMATOPHILUS</i> AND <i>PHOTORHABDUS LUMINESCENS</i> (57) Abstract <p>The invention relates to the identification and isolation of polynucleotide molecules encoding a new class of protein insecticidal toxins which are produced by bacteria from the genera <i>Xenorhabdus</i> and <i>Photorhabdus</i>. The polynucleotide molecules may be incorporated into, for example, insect-specific viruses (including entomopox and nuclear polyhedrosis viruses), bacteria (including <i>Gracilicutes</i>, <i>Firmicutes</i>, <i>Tenericutes</i> and <i>Mendosicutes</i>), protozoa, yeast and plants for control of pest insects.</p>		

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TOXIN GENES FROM THE BACTERIA XENORHABDUS NEMATOPHILUS
AND PHOTORHABDUS LUMINESCENS

Field of the Invention:

5 The present invention concerns the identification and isolation of a new class of protein toxins with specificity for insects, which are produced by bacteria from the genera *Xenorhabdus* and *Photorhabdus*. In addition, the present invention relates to the incorporation of genes encoding this class of toxin into, for example, insect-specific viruses (including entomopox and
10 nuclear polyhedrosis viruses), bacteria (including *Gracilicutes*, *Firmicutes*, *Tenericutes* and *Mendosicutes*), yeast and plants for control of insect pests.

Background of the Invention:

15 Insect pathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* are known to be symbiotically associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus* respectively. It has been observed that these bacteria have the ability to kill a wide range of different insects without the aid of their nematode partners. The present inventors have isolated polynucleotide molecules encoding a new class of protein
20 insecticidal toxins from *Xenorhabdus nematophilus* strain A24 and *Photorhabdus luminescens* strain V16/1.

Disclosure of the Invention:

25 In a first aspect, the present invention provides an isolated polynucleotide molecule encoding an insecticidal toxin, said polynucleotide molecule comprising a nucleotide sequence which substantially corresponds to one of the following;

- (i) the nucleotide sequence shown as SEQ ID NO: 1,
- (ii) the nucleotide sequence shown as SEQ ID NO: 2, and
- 30 (iii) the nucleotide sequence of a portion of (i) or (ii) which encodes an insecticidally-active toxin fragment.

Preferably, said polynucleotide molecule comprises a nucleotide sequence which substantially corresponds to (i) or (ii).

35 In a second aspect, the present invention provides an isolated polynucleotide molecule encoding an insecticidal toxin, said polynucleotide molecule comprising a nucleotide sequence having at least 85%, more

preferably at least 95%, sequence identity to the nucleotide sequence shown as SEQ ID NO: 2.

In a third aspect, the present invention provides an insecticidal toxin, in a substantially pure form, which comprises an amino acid sequence
5 having at least 70% sequence identity to one of the following:

- (i) the amino acid sequence shown as SEQ ID NO: 3,
- (ii) the amino acid sequence shown as SEQ ID NO: 4,
- (iii) the amino acid sequence of an insecticidally-active toxin fragment of (i) or (ii).

10 Preferably, said insecticidal toxin comprises an amino acid sequence having at least 85%, more preferably at least 95%, sequence identity to (i) or (ii). Most preferably, the insecticidal toxin comprises an amino acid sequence substantially corresponding to that defined at (i) or (ii).

In a fourth aspect the present invention provides a recombinant
15 microorganism, the recombinant microorganism being characterised in that it is transformed with and expresses the polynucleotide molecule of the first or second aspects of the present invention.

The microorganisms which may be usefully transformed with the polynucleotide molecule of the first or second aspects of the present
20 invention include bacteria, such as *Escherichia*, *Gracilicutes*, *Firmicutes*, *Tenericutes* and *Mendosicutes*; protozoa and yeast. The microorganism can be transformed by routine methods using expression vectors comprising the toxin-encoding polynucleotide molecule operably linked to a suitable inducible or constitutive promoter sequence.

25 In a fifth aspect, the present invention provides a method of producing an insecticidal toxin, said method comprising:

- (i) culturing a microorganism according to the fourth aspect under conditions suitable for the expression of the toxin-encoding polynucleotide molecule, and
- 30 (ii) optionally recovering the expressed insecticidal toxin.

In a sixth aspect, the present invention provides a recombinant insect-specific virus, the recombinant insect-specific virus being characterised in that it includes within a non-essential region of its genome the polynucleotide molecule of the first or second aspects of the present
35 invention operably linked to a suitable inducible or constitutive promoter sequence.

The recombinant insect-specific virus of the sixth aspect is preferably selected from entomopox and nuclear polyhedrosis viruses. The recombinant virus can be produced by routine methods such as homologous recombination.

5 In a seventh aspect, the present invention provides a method for killing pest insects, said method comprising applying to an area infested with said insects an effective amount of a recombinant microorganism according to the fourth aspect and/or a recombinant virus according to the sixth aspect, optionally in admixture with an acceptable agricultural carrier.

10 In an eighth aspect, the present invention provides a plant transformed with, and capable of expressing, the polynucleotide molecule of the first or second aspects of the present invention.

The plant according to the eighth aspect may be any plant of agricultural, arboricultural, horticultural or ornamental value that is susceptible to damage by feeding pest insects. However, preferably, the plant is selected from plants of agricultural value such as cereals (e.g.; wheat and barley), vegetable plants (e.g.; tomato and potato) and fruit trees (e.g., citrus trees and apples). Other preferred plants include tobacco and cotton.

The plant can be transformed by routine methods including *Agrobacterium* transformation and electroporation. Preferably, the toxin-encoding polynucleotide molecule is operably linked to a suitable inducible or constitutive promoter sequence. Particularly preferred promoter sequences include the cauliflower mosaic virus (CaMV 35 S) promoter element and promoter elements from the sub-clover stunt virus (SCSV).

25 The term "substantially corresponds" as used herein in relation to the nucleotide sequence is intended to encompass minor variations in the nucleotide sequence which due to degeneracy do not result in a change in the encoded protein. Further this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system but in which the variations do not result in a decrease in biological activity of the encoded protein.

30 The term "substantially corresponding" as used herein in relation to the amino acid sequence is intended to encompass minor variations in the amino acid sequence which do not result in a decrease in biological activity of the insecticidal toxin. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, N α -alamino acids.

As used herein the term "insecticidally-active toxin fragment" is intended to encompass fragments of the insecticidal toxin which retain insecticidal activity as may be determined by, for example, the *Galleria mellonella* bioassay described below.

The term "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

The invention will hereinafter be further described by way of reference to the following, non-limiting example and accompanying figures.

Brief description of the accompanying figures:

Figure 1: Nucleotide sequence of the protein coding (sense) strand of the *X. nematophilus* DNA insert of clone toxb4. The translation initiation codon (ATG) at nucleotide position 17-19 and the translation termination codon (TAA) at nucleotide position 1121-1123 are indicated by shaded boxes.

Locations of oligonucleotide sequences used for sequencing primer design are indicated by arrows and a primer name (TOX F1, TOX R3 etc.). Arrows directed left-to-right, positioned above the sequence indicate sense-strand primers, arrows directed right-to-left, positioned below the sequence indicate anti-sense primers.

Figure 2: Deduced sequence of the 368 amino acid toxb4 protein from *X. nematophilus* strain A24, derived by conceptual translation of the long open reading frame commencing at nucleotide position 17 and ending at nucleotide position 1120 of the *toxb4* gene sequence (Fig. 1).

Figure 3: Restriction map of *P. luminescens* V16/1 toxin gene clone showing location of putative toxin protein coding region (solid black box) and direction of transcription (arrow). RI=*EcoRI*, RV=*EcoRV*, H=*Hind III*, S=*Sma I*. Toxin production from clones containing selected restriction fragments is indicated above the restriction map (+, toxin activity; -, no toxin activity).

Figure 4: Nucleotide sequence of the protein coding (sense) strand of the *P. luminescens* Hind III/Sma I DNA fragment. Translation initiation (ATG) and termination (TGA) codons are indicated by shaded boxes. Locations of oligonucleotide sequences used for sequencing primer design are indicated by arrows and a primer name as described in the brief description of Fig. 1. Restriction enzyme sites used for sub-cloning and identification of sequences necessary for toxin activity are underlined and labelled on the figure.

Figure 5: Deduced sequence of the 335 amino acid PlV16tox1 protein from *P. luminescens* strain V16/1, derived by conceptual translation of the long open reading frame commencing at nucleotide position 172 and ending at nucleotide position 1179 of the Hind III/Sma I restriction enzyme fragment (Fig. 4).

Figure 6: Alignment of the nucleotide sequences encompassing the protein open reading frames of the *X. nematophilus* strain A24toxb4 gene and the *P. luminescens* strain V16/1 PlV16tox1 gene using the Gap program of the GCG computer software package. The *X. nematophilus* sequence is the upper line and the *P. luminescens* sequence is the lower line.

Figure 7: Alignment of the deduced protein sequences of the extended open reading frames encoding the *X. nematophilus* A24 toxb4 protein and the *P. luminescens* strain V16/1 PlV16tox1 protein using the Gap program of the GCG computer software package. The *X. nematophilus* sequence is the upper line and the *P. luminescens* sequence is the lower line.

Figure 8: Provides a scheme for expressing and isolating *X. nematophilus* A24toxb4 protein and *P. luminescens* V16/7 PlV16tox1 protein using the IMPACT™ system. The toxin protein is represented schematically as a solid black bar with the first (Met) and last (Ile) amino acids indicated.

Exempl 1**Isolation and Characterisation of Toxin genes from *Xenorhabdus******nematophilus* A24 and *Photorhabdus luminescens*****Construction of recombinant bacterial DNA libraries**

5 High molecular weight genomic DNA was isolated from *Xenorhabdus*
nematophilus strain A24 using the method of Marmur (1961) and from
Photorhabdus luminescens strain V16/1 by the method of Scott *et al.* (1981).
The genomic DNA was partially digested with the restriction enzyme *Sau*
3AI to generate fragments of DNA in the size range 30 to 50 kilobase pairs
10 and dephosphorylated by incubation with the enzyme calf intestinal alkaline
phosphatase. The cosmid cloning vector "Supercos" (Stratagene) was
linearised by digestion with the restriction enzyme *Bam* HI and ligated to the
partially digested bacterial DNA at a vector:genomic DNA ratio of 1:3
according to standard procedures (Maniatis *et al.*, 1982). The ligated DNA
15 was packaged *in vitro* using Gigapack II XL Packaging Extract according to
manufacturer's instructions (Stratagene). The packaged DNA was transfected
into the *Escherichia coli* strain NM554 (F⁻, *recA*, *araD*139, Δ (*ara*, *leu*) 7696,
 Δ *lac* Y74, *galU*⁻, *galK*⁻, *hsr*, *hsm*⁺, *strA*, *mcrA*[-], *mcrA*[-]). Transfected
bacteria were plated onto Luria Bertani (LB) agar medium containing 150µg
20 ml⁻¹ ampicillin, to select for bacteria containing recombinant cosmid clones.

Isolation of an insect toxin gene from *Xenorhabdus nematophilus* strain A24 by functional screening

Cultures of bacteria harbouring individual cosmid clones were grown
overnight at 28°C in LB broth containing 150µg ml⁻¹ ampicillin. The
25 bacterial cultures were treated for 15 minutes with 2mg ml⁻¹ lysozyme to
create cell-free lysates. Five microlitre aliquots of these lysates were injected
into the haemocoel of three *Galleria mellonella* fourth instar larvae. Two
clones with insecticidal activity were identified. Control lysates prepared by
lysozyme treatment of *E. coli* NM554 cells containing non-recombinant
30 Supercos vector possessed no toxin activity in the *Galleria* bioassay.

Characterisation of Toxin Producing Clones

Cosmid DNA from toxin-expressing clones was isolated using a
standard alkaline lysis procedure (Maniatis *et al.*, 1982). Isolated DNA was
analysed by restriction enzyme digestion and agarose gel electrophoresis
35 (Maniatis *et al.*, 1982). Both cosmid clones appeared to contain the same

region of approximately 34.6 kb of *X. nematophilus* genomic DNA. One clone, designated cos149 was chosen for further analysis.

A 7.4 kb *Bam* HI fragment from cos149 was ligated into the plasmid vector pGEM7Z(f)+ (Promega Biotec) and transformed into the *E. coli* strain DH5a (F', F80dlac ZΔ M15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* [r_K , m_K +] *supE44*, *relA1*, *deoR*, Δ[*lacZYA-argF*] U169) using electroporation at 25mF, 200 and 2.5kV in a 0.2cm cuvette in a Bio-Rad Gene Pulser. The resultant sub-clone was designated N8pGEM. Lysates prepared from *E. coli* cells containing the N8pGEM clone contained toxin as determined by the *Galleria* haemolymph injection bioassay.

A set of unidirectional deletion clones was prepared from N8pGEM according to the method of Henikoff (1984) using the Erase-a-base kit (Promega Biotec) and digestion with the enzymes *Cla* I and *Sph* I. Deleted DNA was recircularised by ligation with T4 DNA ligase and transformed into *E. coli* strain DH5α by electroporation as described above. Deletion sub-clones of varying sizes were identified and tested for toxin production using the *Galleria* bioassay. The smallest clone that retained toxin expression (designated tox 1) contained 1.5kb of *X. nematophilus* DNA.

Plasmid DNA from the tox 1 clone was isolated, digested with the restriction enzymes *Sac* I and *Hind* III and directionally deleted with the Erase-a-base kit. A set of deleted clones was identified and tested for toxin production. The smallest clone retaining toxin activity (designated toxb4) contained 1.2kb of *X. nematophilus* DNA. The toxb4 clone was sequenced on both strands with a combination of vector and gene-specific sequencing primers and ABI Prism™ di-deoxy dye-terminator sequencing mix (Applied Biosystems). Plasmid DNA was prepared by a standard alkaline lysis procedure (Maniatis *et al.*, 1982), the double-stranded DNA was sequenced by a thermal cycle sequencing protocol, and sequencing reactions were analysed on an automated DNA sequencer (Applied Biosystems Model 377) according to manufacturer's instructions.

The toxb4 clone contained an insert 1205bp in length (Figure 1) which encoded a protein open reading frame of 368 amino acid residues (Figure 2). Searches of the non-redundant Genbank nucleotide and protein databases were done for the toxb4 nucleotide and deduced protein sequences using the blastn, fasta and blastp, programs for DNA and protein sequences. No

statistically significant similarity was detected between the *X. nematophilus* sequences and sequences present in the databases.

Isolation of a *toxb4* homologue from *Photorhabdus luminescens* strain V16/1

The genomic DNA cosmid library prepared from *P. luminescens* strain V16/1 was screened by nucleic acid hybridisation using the *toxb4* gene as a hybridisation probe. Two hundred clones were grown overnight at 37°C on LB agar plates containing 150 µg ml⁻¹ ampicillin and the resultant bacterial clones were transferred to nylon membrane discs (Colony/Plaque Screen™, NEN DuPont) according to the manufacturer's protocol. Colonies were lysed in situ on the membranes by treatment with 0.5 N NaOH and neutralised with 1.0M Tris-Cl, pH 7.5, and the cosmid DNA was immobilised on the membranes by air drying. Filters were pre-hybridised in a solution consisting of 5X SSPE, 0.2% w/v skim-milk powder, 0.5% w/v SDS and 0.2% mg/ml denatured salmon sperm DNA at 68°C for 3 hours. A hybridisation probe was prepared by radiolabelling approximately 100ng of isolated *toxb4* DNA with 50 µCi α-³²P-dATP by random-primed synthesis using the Gigaprime DNA labelling kit (GPK-1, Bresatec). Filters were incubated with the *toxb4* probe in 5X SSPE, 0.2% w/v skim-milk powder, 0.5% w/v SDS and 0.2% mg/ml denatured salmon sperm DNA at 68°C overnight. Filters were rinsed briefly in 2X SSC, and washed once for 15 min at room temperature in 2X SSC, 0.1% w/v SDS, once at 68°C for 30 min in 0.5X SSC, 0.2% SDS. After a final rinse in 0.5X SSC filters were autoradiographed for 24 hours at -80°C. Three clones that hybridised with the *toxb4* probe were identified. Cultures were grown for each clone and cell lysates were assayed for toxicity using the *Galleria* bioassay. Two clones, designated cos154 and cos160 showed toxin expression. Cosmid DNA was isolated from cos154 and cos160 and analysed by restriction enzyme digestion and Southern blot hybridisation. An 8.5kb *Not* I restriction enzyme fragment that hybridised to the *toxb4* probe was isolated from clone cos160 and sub-cloned into the *Not* I site of the plasmid vector pBC (KS)+ (Stratagene). Further restriction enzyme mapping and bioassay resulted in identification of a 2.4 kb *Eco* RI fragment that contained all the sequences necessary for production of active toxin.

Characterisation of the *P. luminescens* strain V16/1 toxin gene

Three additional sub-clones of the 2.4 kb *Eco* RI fragment were constructed and tested for toxin production (Figure 3). A 1.65kb *Hind* III/*Eco* RI fragment, a 1.39kb *Hind* III/*Sma* I fragment and a 1.44kb *Eco* RV/*Eco* RI

fragment were each ligated into the plasmid vector pBluescript II (KS)+ (Stratagene) and the ligated DNA was transformed into *E. coli* strain DH10B™ (Stratagene) (*F mcrA Δ(mrr-hsdRMS-mcrBC) F80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara, leu)7697 galU galK l rpsL nupG*). Cell lysates were prepared from cultures containing each of these sub-clones and bioassayed by haemocoel injection into *Galleria* larvae. Cultures containing the 1.65kb *Hind* III/*Eco* RI fragment and the 1.39kb *Hind* III/*Sma* I fragment expressed active toxin but cultures containing the 1.44kb *Eco* RV/*Eco* RI fragment were inactive in the bioassay (Fig. 3). Thus, sequences located 5' to the *Eco* RV site of the *P. luminescens* V16/1 *Hind* III/*Eco* RI fragment are required for toxin expression from the plasmid pBluescript II (KS)+, whereas sequences 3' to the *Sma* I site are dispensable. The toxin gene is designated *PlV16tox1* and the toxin protein encoded by this gene is designated PlV16tox1. A strategy was developed for sequencing the 1.39 kb *Hind* III/*Sma* I *P. luminescens* DNA fragment based on internal restriction enzyme sites and custom-synthesised oligonucleotide sequencing primers. The complete sequence of the 1.39 kb *Hind* III/*Sma* I fragment was determined on both strands (Figure 4). Analysis of this DNA sequence identified a single long open reading frame 335 amino acid residues in length (Figure 5).

Comparison of the toxin gene and protein sequences from *X. nematophilus* and *P. luminescens*

The DNA sequences corresponding to the deduced toxin protein open reading frames were compared for the two bacterial species using the 'Gap' program of the GCG software package. The two gene sequences are 83% identical in the coding region (Figure 6) but show no significant similarity in the sequences immediately 5' and 3' of the extended open reading frame. The toxin protein sequences were likewise compared with the 'Gap' program and found to be 75% identical to each other and 86% similar if physico-chemically conservative amino acid differences were taken into consideration (Figure 7). The existence of two extended insertion/deletion variants between the two proteins identifies amino acids that are not essential for toxic activity against *Galleria melonella*.

Example 2:**Distribution of the Toxin gene from *X.nematophilus* A24**

Genomic DNA was prepared from the type strain for each of four identified *Xenorhabdus* species, an additional unclassified *Xenorhabdus* species and six *Photorhabdus luminescens* strains selected to include at least one member of each of the major genetic groups identified by analysis of 16S ribosomal RNA genes (Brunel *et al.*, 1997). The DNA was digested with restriction enzymes, fractionated by agarose gel electrophoresis and transferred to nylon membranes by the Southern blot method (Maniatis *et al.*, 1982). The filters were hybridised with a probe prepared from the *X. nematophilus* A24 *tox4* gene. Hybridisation conditions were selected that would allow sequences with an average identity of approximately 65% to be detected. The results are shown in Table 1.

15 **Table 1**

Bacterial species	Strain	Toxin gene [†]
<i>Xenorhabdus nematophilus</i>	A24	+
<i>Xenorhabdus nematophilus</i>	AN6	+
<i>Xenorhabdus poinarii</i>	G6	-
<i>Xenorhabdus beddingii</i>	Q58	-
<i>Xenorhabdus bovienii</i>	T28	-
<i>Xenorhabdus sp.</i>	K77	-
<i>Photorhabdus luminescens</i>	Hb	-
<i>Photorhabdus luminescens</i>	Hm	-
<i>Photorhabdus luminescens</i>	C1	-
<i>Photorhabdus luminescens</i>	V16	+
<i>Photorhabdus luminescens</i>	C8406	+
<i>Photorhabdus luminescens</i>	K81	+

[†] + indicates presence of hybridising DNA, - indicates absence of hybridisation of toxin gene probe.

Clearly, homologues of the toxin gene from *X.nematophilus* A24 is present in some species of the genus *Xenorhabdus*, and some, but not all isolates of *Photorhabdus luminescens*.

5 **Example 3:**

Activity of Toxin genes cloned into plasmid vectors and transformed into *E. coli*

Active toxin protein was expressed when the A24 toxb4 clone or V16 tox1 genes were inserted into general plasmid vectors of the type pGEM (Promega Biotec) or pBluescript (Stratagene) and the recombinant plasmids transformed into *E. coli*. More specifically, the *X. nematophilus* toxin A24 toxb4 gene was cloned into the plasmid pGEM7z and the *P. luminescens* V16 tox1 gene was cloned into pBluescript SK.

Preparation of cell extract

15 A culture of *E. coli* cells transformed with either a recombinant plasmid containing a toxin gene or a non-recombinant parent plasmid was grown overnight at 37°C in nutrient broth. Lysozyme was added to the culture to a final concentration of 1 mg/ml and the mixture left at room temperature for 30 minutes to lyse the cells. The cleared lysate was used directly for bioassay.

Bioassay

Extracts were bioassayed using the intrahaemocoel injection assay. Ten microlitres of *E. coli* cell lysate were injected into the abdominal region of a *Galleria mellonella* larvae through an intersegmental membrane. Bioassays were done on 10 larvae for each extract and injected larvae were held at 22°C. Mortality was recorded daily. Results are shown in Table 2.

Table 2

Toxin source	Percentage mortality				
	Day 1	Day 2	Day 3	Day 4	Day 5
PIV16tox1	0	20	40	90	100
pBluescript SK (control)	0	10	10	10	20
A24toxb4	10	10	10	100	100
pGEM7z (control)	0	0	10	20	20

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P. luminescens* strain V16/1 kill *G. mellonella* larvae and caused complete mortality of injected individuals five days after injection. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z did not kill the larvae.

Effect of Temperature on Toxicity

Extracts were prepared from *E. coli* cells transformed either with cloned toxin genes or the empty plasmid vector controls and injected into *G. mellonella* larvae as described previously. The injected larvae were maintained at either 20°C or 25°C. Results are shown in Table 3.

Table 3

Toxin source	Temp	Percentage mortality					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
PlV16tox1	20°C	0	10	25	60	90	100
PlV16tox1	25°C	0	0	100	100	100	100
pBluescript SK	20°C	0	0	0	0	0	0
pBluescript SK	25°C	0	5	10	10	15	15
A24toxb4	20°C	5	30	35	65	95	100
A24toxb4	25°C	60	75	100	100	100	100
pGEM7z	20°C	0	5	5	5	5	5
pGEM7z	25°C	0	5	5	5	5	5

Extracts prepared from cells containing either the cloned toxin gene from *X. nematophilus* A24 or the *P. luminescens* V16 toxin gene killed all larvae within three days for larvae held at 25°C or by six days for larvae maintained at 20°C following injection. Control extracts prepared from cells containing only the cloning vectors pBluescript or pGEM7z did not cause significant larval mortality.

Example 4:**Toxin Activity Against Different Insect Species****(1) *Helicoverpa armigera* (Lepidoptera:Noctuidae)****Bioassay**

5 Extracts were bioassayed using the intrahaemocoel injection assay. Ten microlitres of *E. coli* cell lysate were injected into the abdominal region of fourth instar *Helicoverpa armigera* larvae through an intersegmental membrane. Bioassays were done on 24 larvae for each extract and injected animals were held at 27°C. Mortality was recorded daily. Results are shown
10 in Table 4.

Table 4

Toxin source	Percentage mortality			
	Day 1	Day 2	Day 3	Day 4
PIV16tox1	38	71	87	91
pBluescript SK	4	4	8	8
A24toxb4	50	87	91	91
pGEM7z	0	0	0	0

15 Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P. luminescens* strain V16/1 caused significant mortality to injected larvae within 24 hours after injection. All larvae died by 4 days following the injection, with the exception of a small number of "escapees" that resulted
20 from leakage of injected material upon removal of the injection needle. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z had no significant effect on *H. armigera* larvae.

(2) *Plodia interpunctella* (Lepidoptera:)**Bioassay**

25 Extracts were bioassayed using the intrahaemocoel injection assay. Five microlitres of *E. coli* cell lysate were injected into the abdominal region of a final instar *Plodia interpunctella* larva through an intersegmental membrane. Bioassays were done on 20 wandering-stage larvae for each

extract and injected animals were held at 26°C. Mortality was recorded daily. Results are shown in Table 5.

Table 5

5

Toxin source	Percentage mortality		
	Day 1	Day 2	Day 3
PIV16tox1	20	90	100
pBluescript SK	0	0	0
A24tox4	75	95	100
pGEM7z	0	5	5

10 Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P. luminescens* strain V16/1 caused significant mortality to injected larvae within 24 hours after injection. All larvae had died within 3 days. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z had no significant effect on survival of *P. interpunctella* larvae.

(3) *Lucilia cuprina* (Diptera: Calliphoridae) Adults

15

Bioassay

20 Extracts were bioassayed using the intrahaemocoel injection assay. Five microlitres of *E. coli* cell lysate were injected into the abdomen of a 3 day old *Lucilia cuprina* female fly through an intersegmental membrane. Bioassays were done on 20 flies for each extract and injected animals were held at 25°C. Mortality was recorded daily. Results are shown in Table 6.

Table 6

Toxin source	Percentage mortality			
	Day 1	Day 2	Day 3	Day 4
PIV16tox1	55	65	85	100
pBluescript SK	20	25	25	25
A24tox4	55	75	85	100
pGEM7z	30	60	65	65

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P. luminescens* strain V16/1 caused significant mortality to injected flies within 24 hours of injection. All flies died by 4 days after injection. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z also caused significant mortality to the *L. cuprina* flies in the first 48 hours following injection. After this control mortality stabilised, there was no further deaths for the remainder of the test period. Additional experiments with saline injections showed that the early mortality in the control group resulted from physical damage to the flies as a result of the injection process.

(4) *Lucilia cuprina* (Diptera: Calliphoridae) Larvae
Bioassay

Extracts were bioassayed using the intrahaemocoel injection assay. Five microlitres of *E. coli* cell lysate were injected into the abdominal cavity of wandering-stage final instar *Lucilia cuprina* larvae through an intersegmental membrane. Bioassays were done on 20 larvae for each extract and injected animals were held at 25°C. Mortality was recorded daily. Results are shown in Table 7.

Table 7

Toxin source	Percentage mortality			
	Day 1	Day 2	Day 3	Day 4
PIV16tox1	35	45	75	80
pBluescript SK	25	30	30	30
A24toxb4	10	35	90	95
pGEM7z	15	20	20	25

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P. luminescens* strain V16/1 caused significant mortality to injected larvae within 48 hours of injection. All larvae died by 4 days after injection, with the exception of a small number of "escapees" resulting from leakage at the time of needle withdrawal as previously described for *H. armigera*. As with

the *L. cuprina* adults, extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z caused significant mortality to the *L. cuprina* larvae in the first 48 hours following injection. After this, control mortality stabilised and there were no further deaths in this group of larvae for the remainder of the test period. As described above, experiments with saline injections showed that this early mortality in the control group resulted from physical damage to the larvae as a result of the injection process.

(5) *Aphis gossypii* (Hemiptera:Aphididae) Nymphs
Bioassay

Extracts were prepared from *E. coli* cells containing either the *X. nematophilus* toxin gene or the empty plasmid vector pGEM7z. The extracts were incorporated into a defined liquid diet at a concentration of 10% by volume and aphids were provided *ad libitum* access to diet for a period of five days. Results are shown in Table 8.

Table 8

Treatment	% Mortality at day 5	Average Number of Moult
Control [†]	10	1.9
pGEM7z extract	0	2
A24toxb4 extract	90	0.6

[†] an additional treatment consisting of diet supplemented with lysozyme at the same final concentration used to prepare the *E. coli* cell extracts was included as a control for any potential effects of the lysozyme.

The *X. nematophilus* A24 toxin effectively blocked growth as seen from the reduction in the number of nymphal moults, and by five days had killed most of the larvae. Thus, the *X. nematophilus* A24 toxin was orally insecticidal to *Aphis gossypii*.

Exempl 5:**Expression and Purification of the Full-length Toxin Protein from *X.nematophilus***

Further characterisation of the properties of the toxins encoded by the cloned genes from *X. nematophilus* A24 and *P. luminescens* V16/1 required expression of the full-length protein in a format that allowed for affinity purification of the toxin. This was achieved by expressing the full-length toxin as a fusion protein in which the fusion partner was used for affinity selection, and the toxin domain was cleaved off chemically after the purification stage. A suitable expression and purification system is the IMPACT™ system (New England Biolabs) in which the toxin open reading frame is cloned at the 5' end of a self-splicing intein coding sequence fused to a short DNA sequence encoding a chitin binding domain.

Recombinant plasmids containing both the *X. nematophilus* A24 toxin and the *P. luminescens* V16/1 toxin genes were prepared in the IMPACT™ vector pCYB3 (Figure 8). Preparation of these constructs required the engineering of a unique restriction enzyme site at each end of the toxin open reading frame that enabled in-frame insertion of the toxin gene into the expression vector such that translation began at the Methionine initiation codon of the toxin protein and a cleavage site for protein splicing was placed immediately adjacent to the final residue of the toxin open reading frame. Expression of the fusion proteins in *E. coli*, preparation of bacterial cell extracts, affinity isolation of the fusion proteins on chitin cellulose columns, on-column DTT-mediated cleavage of the fusion proteins and elution of the purified toxin proteins were all performed according to the manufacturer's instructions (IMPACT™ system manual, New England Biolabs)

For both toxin constructs a major protein product of the expected size (approximately 40 kDa) was detected by SDS polyacrylamide gel electrophoretic analysis of the column eluate. The preparations contained several other proteins but these comprised less than 10% of the total protein present in the samples as determined by Coomassie blue staining of the polyacrylamide gels. Approximately 750µg of PlV16tox1 toxin and 1.5mg of A24tox4 toxin were isolated from one litre of *E. coli* broth cultures. Purified proteins were dialysed against phosphate-buffered saline and simultaneously concentrated by diafiltration to a final concentration of approximately

1mg/ml on Millipore spin cartridges with a membrane nominal molecular weight cut-off of 10kDa according to manufacturer's instructions (Millipore).

Example 6:

Biological Activity of Purified Toxin Proteins

Bioassay

The activity of the purified *X. nematophilus* and *P. luminescens* toxins were determined by intra-haemocoel injection bioassay on *Galleria mellonella* and *Helicoverpa armigera* larvae as described above. The toxin protein preparations were diluted in phosphate-buffered saline and 10ml of protein solution was injected into each larva. Ten larvae were injected for each protein concentration and mortality was recorded at 12 hour intervals for six days after injection. Proteins were tested over a dose range from 1 nanogram (10^{-9} g) to 1 microgram (10^{-6} g) of protein per larva. An inert protein, *E. coli* maltose binding protein, was prepared in the IMPACT™ system, purified and concentrated according to the same methods used for the two toxin proteins. The purified maltose binding protein was used as a control for these experiments. The maltose binding protein did not cause larval mortality at any of the quantities tested. The results are shown in Tables 9 to 12.

Table 9:

Effect of purified PLV16 tox1 toxin on *G. mellonella* larvae

Protein Injected	Percentage Mortality								
	Day2 am	Day2 pm	Day3 am	Day3 pm	Day4 am	Day4 pm	Day5 am	Day5 pm	Day6 am
1ng	0	0	0	0	0	0	0	0	0
10ng	0	0	0	0	0	0	0	0	10
20ng	0	10	10	20	20	20	20	30	30
100ng	0	0	30	40	60	70	80	80	100
200ng	0	0	44	56	56	78	100	100	100
1000ng	20	20	60	60	100	100	100	100	100

Table 10:

Effect of purified A24 toxb4 toxin on *G. mellonella* larvae

Protein Injected	Percentage Mortality								
	Day2 am	Day2 pm	Day3 am	Day3 pm	Day4 am	Day4 pm	Day5 am	Day5 pm	Day6 am
1ng	0	0	0	0	0	0	0	0	0
10ng	0	0	0	0	0	0	0	0	0
20ng	0	0	10	20	20	40	60	70	80
100ng	10	10	20	30	30	50	90	100	100
200ng	0	0	0	0	50	70	70	90	100
1000ng	0	0	0	10	60	80	100	100	100

5

Table 11:

Effect of purified PLV16 tox1 toxin on *H. armigera* larvae

Protein Injected	Percentage Mortality							
	Day1/am	Day1/pm	Day2/am	Day2/pm	Day3/am	Day3/pm	Day4/am	Day4/pm
1ng	0	0	0	0	0	0	0	0
10ng	0	0	0	0	0	0	0	0
20ng	0	10	10	10	10	10	10	10
100ng	30	30	50	50	60	70	70	70
200ng	0	0	80	80	80	80	80	80
1000ng	22	67	100	100	100	100	100	100

Table 12:

10

Effect of purified A24 toxb4 toxin on *H. armigera* larvae

Protein Injected	Percentage Mortality							
	Day1/am	Day1/pm	Day2/am	Day2/pm	Day3/am	Day3/pm	Day4/am	Day4/pm
1ng	0	0	0	0	0	0	0	0
10ng	0	30	50	70	90	90	90	90
20ng	0	30	50	80	90	90	90	90
100ng	0	20	80	100	100	100	100	100
200ng	0	30	90	100	100	100	100	100
1000ng	20	60	100	100	100	100	100	100

Both the *X. nematophilus* A24 toxin and the *P. luminescens* V16/1 toxin killed a high percentage of larvae after a single injection of at least 20ng of toxin protein per larva. Mortality was dependent on toxin type and concentration. *H. armigera* was sensitive to small quantities of *X. nematophilus* A24 toxin with high mortality at 10-20ng of toxin per larva, but was less sensitive to *P. luminescens* V16/1 toxin where significant mortality was observed only for quantities greater than 20ng of protein per larva. A similar pattern of sensitivity was observed for *G. mellonella* larvae. The time taken to kill the larvae of either species was not strongly dependent on the time since toxin injection, although larger amounts of toxin killed more quickly. However, at all quantities greater than, or equal to 20ng per larva the insects were effectively dead, because the *H. armigera* larvae ceased feeding and *G. mellonella* larvae were unable to spin cocoon silk.

Thus, the proteins encoded by the A24 *tox*b4 genes of *X. nematophilus* and the PlV16 *tox*1 gene of *P. luminescens* encode toxin proteins that are effective insecticides, especially of lepidopterous larvae including *G. mellonella*, *H. armigera* and *P. interpunctella*, when delivered into insect haemocoel.

Example 7:

Effect of Purified Toxin on Insect Cells in Culture

The purified *X. nematophilus* A24 toxin and *P. luminescens* V16/1 toxin and the maltose binding protein control were each tested for their effects on the growth and viability of insect cells in tissue culture. A sample of 10^4 cells in the appropriate culture medium was mixed with the test proteins at several different concentrations and seeded into the wells of a 96-well tissue culture plate. Cells were allowed to grow for 24 hours at 25°C and cells were counted in a haemocytometer and assessed visually for cell lysis. The results are shown in Table 13.

Table 13

Cell Line	Treatment		Cells/well
	Toxin	Concentration n µg/ml	
Schneider 2	PIV16tox1	0	4.1×10^4
	"	0.001	ND [†]
	"	0.1	4.1×10^4
	"	1	4.6×10^4
Schneider 2	A24toxb4	0	3.7×10^4
	"	0.001	ND
	"	0.1	3.6×10^4
	"	1	3.4×10^4
High-Fives	PIV16tox1	0	3.8×10^4
	"	0.001	ND
	"	0.1	3.9×10^4
	"	1	2.9×10^4
High-Fives	A24toxb4	0	8.2×10^4
	"	0.001	7.1×10^4
	"	0.1	2.5×10^4
	"	1	2.5×10^4
Sf9	PIV16tox1	0	3.6×10^4
	"	0.001	4.3×10^4
	"	0.1	7×10^3
	"	1	6×10^3
Sf9	A24toxb4	0	4.7×10^4
	"	0.001	1×10^4
	"	0.1	5×10^3
	"	1	6.5×10^3

[†]ND: cell numbers not determined

For all cell lines, at all protein concentrations tested the maltose binding protein control had no effect on cell growth or viability. Neither of the toxin proteins had any significant effect on cell growth or viability for the *Drosophila melanogaster* Schneider 2 cell line. The *X. nematophilus* A24 toxin caused significant cell growth inhibition and cytotoxicity to the lepidopteran High-Five cell line at concentrations above 0.1µg/ml. The *P. luminescens* V16 toxin caused slight growth inhibition only at the highest concentration tested of 1µg/ml. The *X. nematophilus* A24 toxin caused significant cell growth inhibition and cytotoxicity to the lepidopteran Sf9 cell line at concentrations above 0.001µg/ml, and the *P. luminescens* V16 toxin was toxic to this cell line at concentrations of 0.1µg/ml and higher. Thus, toxins of this family exhibit growth inhibitory and cytotoxic activity against insect cells in tissue culture, especially cell lines of lepidopteran origin. Similar tests with a mouse hybridoma cell line demonstrated slight growth inhibition only by the *X. nematophilus* A24 toxin, and only at the highest concentration tested of 1µg/ml.

As will be appreciated by persons skilled in this field, the present invention provides a new class of toxins useful for genetically engineering a wide range of biological systems which will thus become more useful for control of pest insects detrimental to agricultural, aquatic and forest industries. This new class of toxin may be purified by one or more methods of protein purification well known in the art. Insecticidal fragments may be generated from the purified toxin using, for example, cleavage with trypsin or cyanogen bromide.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Sequence Listings:

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and *Photorhabdus luminescens*

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aatggagatc caaagtgttg gaactcatta ccaaaatata taacctgatt tatagtagtg 1140
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1205

SEQ ID NO: 6

<211> 1388

<212> DNA

<213> *Photorhabdus luminescens*

<400> 6

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ctgccagaaa tttcatagct caaataaaca ttaacataat ggagaaatat aatggttata 180
caattaacac ctgatgatag aagtggatat ccaccggtg aaaagcaaat agcaggagat 240
atagtacgta tactaaactt taagcaaaca gatgagggtc atacagcatc atatggaatt 300
gaatatcgag ctaagaaaat aatattagct tacgctttgg ctgtaagtgg tattcataat 360
gtatctaaac ttctgatga ctattataag aataaagaga ctgctgagag aatttatcaa 420
gaatatatgt ctaatctttc atctgcacta ttaggtgaaa atggtgatca aatttctaaa 480
gatatggcaa atggttttta taagaatgaa ctggattttg aaggtcaata tcctcaaaac 540
at ttgggaatg ttctgagct tgaaaaataa ccattgagtg cttattcaga tgacgataaa 600
ttattagcac tatatttttt ctctgtacag gaaattccac tggaggaaaa tcaacaatca 660
aatgccgcaa gattttttta attaatgat ttcttattta ccttatctgc tgtaacttca 720
ctgggaagga ggattttttc aaaaaacttt tacaatggat tagaggctaa atcattagag 780
aattatatgt agagaaaaaa acttttctaaa cctttctttc gaccaccgca gagattacct 840
gatggcagaa taggttattt ggctggacca acagaagcgc ctaaattggag agtgagtttt 900
aaagaactta aaaataacaa atctaggaat ggattttcta atatggaag ggctgcaaaa 960
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ctaccaagaa aatatatacc ctatggattt caagatggat cgcgggcgga agggagcgaa 1380
tccccggg 1388

Claims:

1. An isolated polynucleotide molecule encoding an insecticidal toxin,
said polynucleotide molecule comprising a nucleotide sequence which
5 substantially corresponds to one of the following:
 (i) the nucleotide sequence shown as SEQ ID NO: 1;
 (ii) the nucleotide sequence shown as SEQ ID NO: 2; and
 (iii) the nucleotide sequence of a portion of (i) or (ii) which encodes
an insecticidally-active toxin fragment.
- 10 2. An isolated polynucleotide molecule according to claim 1, wherein
said polynucleotide molecule comprises a nucleotide sequence which
substantially corresponds to that shown as SEQ ID NO: 1.
- 15 3. An isolated polynucleotide molecule according to claim 1, wherein
said polynucleotide molecule comprises a nucleotide sequence which
substantially corresponds to that shown as SEQ ID NO: 2.
- 20 4. An isolated polynucleotide molecule encoding an insecticidal toxin,
said polynucleotide molecule comprising a nucleotide sequence having at
least 85% sequence identity to the nucleotide sequence shown as SEQ ID
NO: 2.
- 25 5. An isolated polynucleotide molecule according to claim 4, wherein
said polynucleotide molecule comprises a nucleotide sequence having at
least 95% sequence identity to the nucleotide sequence shown as SEQ ID
NO: 2.
- 30 6. An insecticidal toxin, in a substantially pure form, which toxin
comprises an amino acid sequence having at least 70% sequence identity to
one of the following:
 (i) the amino acid sequence shown as SEQ ID NO: 3;
 (ii) the amino acid sequence shown as SEQ ID NO: 4;
 (iii) the amino acid sequence of an insecticidally-active toxin
35 fragment of (i) or (ii).

7. An insecticidal toxin according to claim 6, wherein the toxin comprises an amino acid sequence having at least 85% sequence identity to that shown as SEQ ID NO: 3.
- 5 8. An insecticidal toxin according to claim 6, wherein the toxin comprises an amino acid sequence having at least 95% sequence identity to that shown as SEQ ID NO: 3.
- 10 9. An insecticidal toxin according to claim 6, wherein the toxin comprises an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 3.
- 15 10. An insecticidal toxin according to claim 6, wherein the toxin comprises an amino acid sequence having at least 85% sequence identity to that shown as SEQ ID NO: 4.
- 20 11. An insecticidal toxin according to claim 6, wherein the toxin comprises an amino acid sequence having at least 95% sequence identity to that shown as SEQ ID NO: 4.
- 25 12. An insecticidal toxin according to claim 6, wherein the toxin comprises an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 4.
- 30 13. A recombinant microorganism, the microorganism being characterised in that it is transformed with and expresses a polynucleotide molecule according to any one of the claims 1 to 5.
14. A recombinant microorganism according to claim 12, wherein the microorganism is selected from bacteria, protozoa and yeast.
- 35 15. A method of producing an insecticidal toxin, said method comprising:
(i) culturing a microorganism according to claim 13 or 14 under conditions suitable for the expression of the toxin-encoding polynucleotide molecule; and
(ii) optionally recovering the expressed insecticidal toxin.

16. A method for killing pest insects, said method comprising applying to an area infested with said insects an effective amount of a recombinant microorganism according to claim 13 or 14 optionally in admixture with an acceptable agricultural carrier.

17. A recombinant insect-specific virus, the recombinant insect-specific virus being characterised in that it includes within a non-essential region of its genome a polynucleotide molecule according to any one of claim 1 to 5 operably linked to a suitable inducible or constitutive promoter sequence.

18. A method for killing pest insects, said method comprising applying to an area infested with said insects an effective amount of a recombinant virus according to claim 17 optionally in admixture with an acceptable agricultural carrier.

19. A plant transformed with, and capable of expressing, the polynucleotide molecule according to any one of claims 1 to 5.

1 ATAATGGGAA AGTAC[**ATG**] TTATTAAACC CGTAACAACT CCGAGTGTAA
 51 TACAATTAAC GCCTGATGAT AGAGTAACGC CTGATGATAA AGGTGAATAT
 101 CAACCCGTTG AAAAGCAAAT AGCGGGAGAT ATAATACGTG TACTAGAATT
 151 CAAGCAAACA AATGAAAGTC ATACAGGATT GTATGGAATT GCATATCGAG
 201 CTAAGAAAGT AATAATAGCA TATGCTTAG TOX F2 CGGTAAGTGG TATTCATAAT
 251 GTCTCTCAAC TTCCAGAAGA CTATTATAAA AATAAGGATA ACACAGGTAG
 301 AATTTATCAA GAATACATGT CTAATCTTTT ATCTGCACTA TTGGGTGAGA
 351 ATGGTGATCA AATTTCTAAA GATATGGCAA ATGATTTTAC CCAGAACGAA
 401 CTGGAGTTTG GAGGTCAACG TCTTAAAAAT ACCTGGGATA TTCCTGATCT
 451 TGAGAATAAA CTATTGGAAG ATTATTCAGA TGAAGATAAA TTATTAGCAC
 501 TATATTTCTT TGCTTCACAA GAACTTCCAA TOX F1 TGGAGGCAAA TCAACAATCA
 551 AATGCAGCAA ATTTTTTTTAA AGTAATTGAT TTTTACTTA TOX R3 TCTTATCTGC
 601 TGTAACATCA CTGGGAAAAA GGATTTTTTC AAAAAATTTT TACAATGGTC
 651 TAGAAACTPA ATCATTAGAG AATTATATTG AGAGAAAAAA ACTTTCTAAA
 701 CCTTCTTTC GACCACCGCA GAAGTTACCT TOX F3 GATGGCAGAA CAGGCTACTT
 751 GGCCGGTCCA ACAAAGCGC CTAAATTGCC AACAACGTCT TOX R4 TCTACAGCAA
 801 CAACGTCTAC AGCAGCTTCA TCTAATTGGA GAGTTAGTTT GCAAAAACCT
 851 AGAGATAACC CATCCAGAAA TACATTTATG AAAATGGATG ATGCTGCAAA
 901 ACGAAAATAT AGTTCATTTA TAAAAGAGGT ACAAAGGGT AATGATCCAC
 951 GTGCAGCAGC AGCAAGTATT GGTACAAAAA GCGGCAGTAA CTTCGAAAAA
 1001 CTGCAAGGTA GAGATTTATA TAGTATAAGA A24AC1 CTAAGCCAAG AACACAGGGT
 1051 AACATTCTCC ATAAATAATA CTGACCAAAT AATGGAGATC CAAAGTGTTG
 1101 GAACTCATTA CAAAATATA **TAA** CCTGATT TATAGTAGTG ATAAGACGTA
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 1201 CTCAG

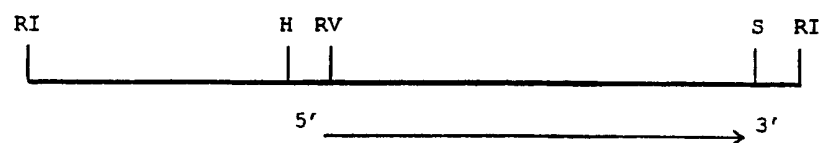
FIGURE 1 .

1 MVIKPVTTPS VIQLTPDDR V TPDDKGEYQP VEKQIAGDII RVLEFKQTNE
51 SHTGLYGIAY RAKKVIIAYA LAVSGIHNVS QLPEDYYKNK DNTGRIYQY
101 MSNLLSALLG ENGDQISKDM ANDFTQNELE FGGQRLKNTW DIPDLENKLL
151 EDYSDEDKLL ALYFFASQEL PMEANQQSNA ANFFKVIDFL LILSAVTSLG
201 KRIFSKNFYN GLETKSLENY IERKKLSKPF FRPPQKLPDG RTGYLAGPTK
251 APKLPTTSST ATTSTAASN WRVSLQKLRD NPSRNTFMKM DDAAKRKYSS
301 FIKEVQKGND PRAAAASIGT KSGSNFEKLQ GRDLYSIRLS QEHRVTFESIN
351 NTDQIMEIQS VGTHYQNI

FIGURE 2

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	Restriction Enzyme Fragment	Toxin Activity
<i>Eco</i> RI/ <i>Eco</i> RI	—————	+
<i>Hind</i> I	—————	+
<i>Hind</i> III/ <i>Sma</i> I	—————	+
<i>Eco</i> RV/ <i>Eco</i> RI	—————	—



—————
250 bp



FIGURE 3

1 AAGCTTGCTA ATAATTCTTG CGTAAGTTAA TTTTACATTG AAATTAACGC
Hind III
51 TTA AAAAGGCC AGGGAAAAC TATATTTAA AGTTGAAATT TATATTAGTA
101 GCGACAAATT GCGGAGTTTT CTGCCAGAAA TTTCATAGCT CAAATAAAACA
151 TTAACATAAT GGAGAAATAT AATGGTTATA CAATTAACAC CTGATGATAG
201 AAGTGGATAT CCACCCGTTG AAAAGCAAAT AGCAGGAGAT ATAGTACGTA
Eco RV
251 TACTAAACTT TAAGCAAACA GATGAGGGTC ATACAGCATC ATATGGAATT
301 GAATATCGAG CTAAGAAAAT AATATTAGCT TACGCTTTGG CTGTAAGTGG
AC4R
351 TATTCATAAT GTATCTAAAC TTCCTGATGA CTATTATAAG AATAAAGAGA
401 CTGCTGAGAG AATTTATCAA GAATATATGT CTAATCTTTC ATCTGCACTA
451 TTAGGTGAAA ATGGTGATCA AATTTCTAAA GATATGGCAA ATGGTTTTTA
AC2F
501 TAAGAATGAA CTGGATTTTG AAGGTCAATA TCCTCAAAC ATTTGGAATG
551 TTCCTGAGCT TGAAAATAAA CCATTGAGTG CTTATTCAGA TGACGATAAA
AC7R
601 TTATTAGCAC TATATTTTTT CTCTGTACAG GAAATCCAC TGGAGGAAAA
651 TCAACAATCA AATGCCGCAA GATTTTTTAA ATTAATTGAT TTCTTATTTA
701 CCTTATCTGC TGTAACCTCA CTGGGAAGGA GGATTTTTTC AAAAACTTT
751 TACAATGGAT TAGAGGCTAA ATCATTAGAG AATTATATTG AGAGAAAAA
AC6F
801 ACTTTCTAA CTTTCTTTTC GACCACCGCA GAGATTACCT GATGGCAGAA
851 TAGGTTATTT GGCTGGAAC ACAGAAGCGC CTAATGGAG AGTGAGTTTT
AC5R
901 AAAGAACTTA AAAATAACAA ATCTAGGAAT GGATTTTCTA ATAIGGAAGG
951 GGCTGCAAAA CAAAAGTATA GTTCATTTAT AAAAGAGGTA CAAAAGGGTA
1001 ACGCTCCACA GACAGCAGCG AAAAGTATTG GTACAGCCAG TGGCAGTAAC
1051 CTGAAAAAAT TGCCGAATAA TTTATATAGT GTGAGGCTAA GCCAAAAAGA
AC3F
1101 CAGGGTAACC TTTACTCAAA ATGATACTGA CAATACAATG ACGGTTCTATA
AC8R
1151 GTGTGGAAC TCATTATAAA AATATATGAT GAGTAATCTC TGACTTCGAT
1201 TGACAGAGCA TTTTAAAGCT CTCATTTTCT CAACGGGAGT CTCATAAGGC
1251 GTTTTACTTT TCAAGCCACT ATGTGGTCTG TGATAATTGT AAAACGCCTT
1301 CTTTTAGCCA ATACACTTTA CTACCAAGAA AATATATACC CTATGGATTT
V16AC1
1351 CAAGATGGAT CGCGCGGCA AGGGAGCGAA TCCCCGGG
Sma I

FIGURE 4

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1 MVIQLTPDDR SGYFPVEKQI AGDIVRIILNF KQTDEGHTAS YGIEYRAKKI
51 ILAYALAVSG IHNVSKLPDD YYKNKETAER IYQEYMSNLS SALLGENGDQ
101 ISKDMANGFY KNELDFEGQY PQNIWNPVEL ENKPLSAYS DDKLLALYFF
151 SVQEIPLEEN QQSNAARFFK LIDFLFTLSA VTSLGRRIFS KNFYNGLEAK
201 SLENYIERKK LSKPFFRPPQ RLPDGRIGYL AGPTEAPKWR VSFKELKNNK
251 SRNGFSNMEG AAKQKYSSFI KEVQKGNAPQ TAAKSIGTAS GSNLEKLPNN
301 LYSVRLSQKD RVTFTQNDTD NTMTVHSVGT HYKNI

FIGURE 5

17 ATGGTTATTAAACCCGTAACAACTCCGAGTGTAAATACAATTAACGCCTGA 66
172ATGGT 176
67 TGATAGAGTAACGCCTGATGATAAAGGTGAATATCAACCCGTTGAAAAGC 116
177 TATACAATTAACACCTGATGATAGAAGTGGATATCCACCCGTTGAAAAGC 226
117 AAATAGCGGGAGATATAATACGTGTACTAGAATTCAAGCAAACAAATGAA 166
227 AAATAGCAGGAGATATAGTACGTATACTAAACTTTAAGCAAACAGATGAG 276
167 AGTCATACAGGATTGTATGGAATTGCATATCGAGCTAAGAAAGTAATAAT 216
277 GGTATACAGCATCATATGGAATTGAATATCGAGCTAAGAAAATAATATT 326
217 AGCATATGCTTTAGCGGTAAGTGGTATTCATAATGTCTCTCAACTTCCAG 266
327 AGCTTACGCTTTGGCTGTAAGTGGTATTCATAATGTATCTAAACTTCCTG 376
267 AAGACTATTATAAAAATAAGGATAACACAGGTAGAATTTATCAAGAATAC 316
377 ATGACTATTATAAGAATAAAGAGACTGCTGAGAGAATTTATCAAGAATAT 426
317 ATGTCTAATCTTTTATCTGCACTATTGGGTGAGAATGGTGATCAAATTC 366
427 ATGTCTAATCTTTCATCTGCACTATTAGGTGAAAATGGTGATCAAATTC 476
367 TAAAGATATGGCAAATGATTTTACCCAGAACGAACTGGAGTTTGGAGGTC 416
477 TAAAGATATGGCAAATGGTTTTTATAAGAATGAACTGGATTTTGAAGGTC 526
417 AACGTCTTAAATAACCTGGGATATTCCTGATCTTGAGAATAAACTATTG 466
527 AATATCCTCAAAACATTTGGAATGTTCTGAGCTTGAAAAATAACCATTG 576
467 GAAGATATTCAGATGAAGATAAATTATTAGCACTATATTTCTTTGCTTC 516
577 AGTGCTTATTCAGATGACGATAAATTATTAGCACTATATTTTCTCTGT 626
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627 ACAGGAAATTCCTGAGGAAATCAACAATCAAATGCCGCAAGATTT 676
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727 AGGAGGATTTTTTCAAAAATTTTACAATGGATTAGAGGCTAAATCATT 776
667 AGAGAATTATATTGAGAGAAAAAACTTTCTAAACCTTTCTTTGACCAC 716
777 AGAGAATTATATTGAGAGAAAAAACTTTCTAAACCTTTCTTTGACCAC 826
717 CGCAGAAGTTACCTGATGGCAGAACAGGCTACTTGCCCGTCCAACAAA 766
827 CGCAGAGATTACCTGATGGCAGAATAGGTTATTGGCTGGACCAACAGAA 876

Figure 6

```
767 GCGCCTAAATTGCCAACAACGTCTTCTACAGCAACAACGTCTACAGCAGC 816
      |||||
877 GCGCCTAAA..... 885
817 TTCATCTAATTGGAGAGTTAGTTTGCAAAAACCTTAGAGATAACCCATCCA 866
      ||||| |||| | | |||| | |||| |||
886 .....TGGAGAGTGAGTTTAAAGAACTTAAAAATAACAAATCTA 925
867 GAAATACATTTATGAAAATGGATGATGCTGCAAAACGAAAATATAGTTCA 916
      |||| |||| | ||||| | ||||| ||| |||||
926 GGAATGGATTTTCTAATATGGAAGGGGCTGCAAAACAAAAGTATAGTTCA 975
917 TTTATAAAAGAGGTACAAAAGGGTAATGATCCACGTGCAGCAGCAGCAAG 966
      ||||| ||||| ||||| ||||| |||
976 TTTATAAAAGAGGTACAAAAGGGTAACGCTCCACAGACAGCAGCGAAAAG 1025
967 TATTGGTACAAAAGCGGCAGTAACTTCGAAAACTGCAAGGTAGAGATT 1016
      ||||| || ||||| | |||| ||| |||
1026 TATTGGTACAGCCAGTGGCAGTAACCTGGAAAAATTGCCGAATA...ATT 1072
1017 TATATAGTATAAGACTAAGCCAAGAACACAGGGTAACATTCTCCATAAAT 1066
      ||||| | || ||||| || ||||| || | ||||
1073 TATATAGTGTGAGGCTAAGCCAAAAGACAGGGTAACCTTTACTCAAAAT 1122
1067 AATACTGACCAAATAATGGAGATCCAAAGTGTTGGAACCTATTACCAAAA 1116
      ||||| | |||| | | || ||||| ||||| |||
1123 GATACTGACAATACAATGACGGTTCATAGTGTTGGAACCTATTATAAAAA 1172
1117 TATA... 1120
      |||
1173 TATATGA 1179
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Figure 6 continued

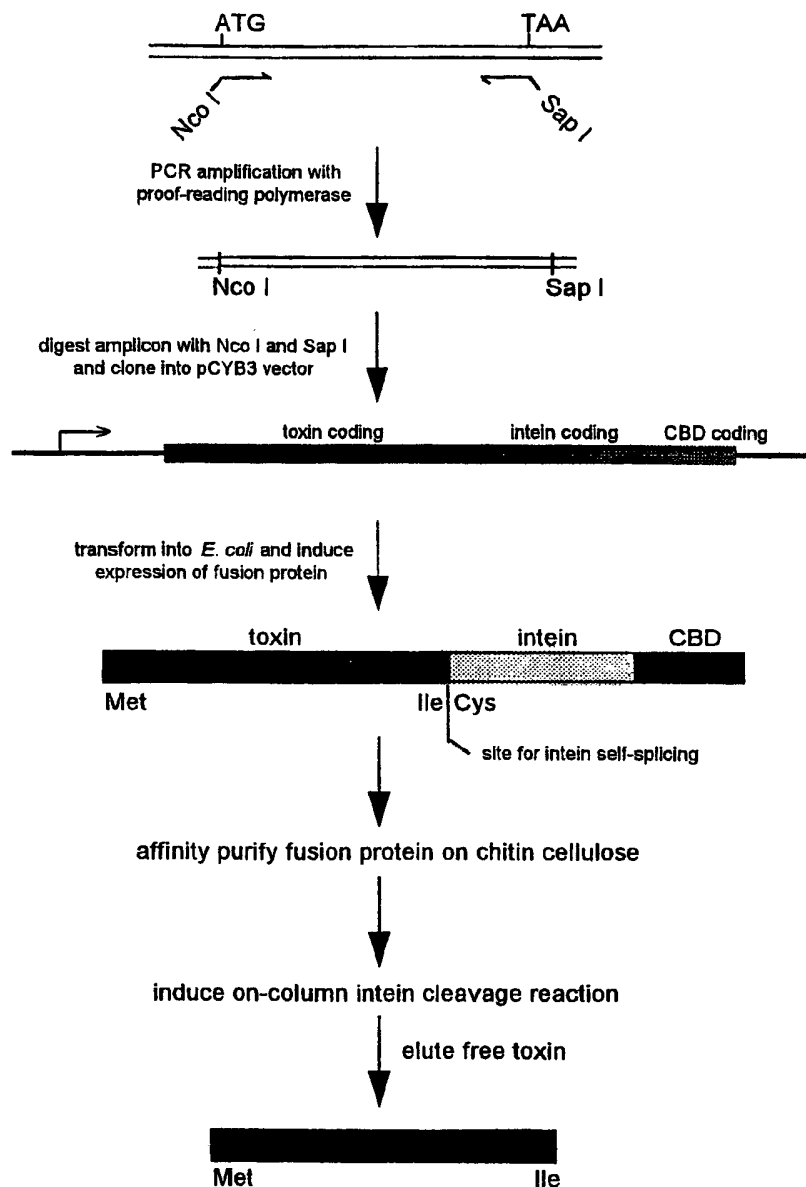
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1 MVIKPVTTSPVIQLTPDDRVTTPDDKGEYQPVVEKQIAGDIIRVLEFKQTNE 50
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1 .....MVIQLTPDDR.....SGYPPVEKQIAGDIVRILNFKQTDE 35
51 SHTGLYGIAYRAKKVVIAYALAVSGIHNVSQLPEDYYKNKDNTGRIYQEY 100
   :||: |||:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|
36 GHTASYGIEYRAKKIILAYALAVSGIHNVSQLPEDYYKNKETAERIYQEY 85
101 MSNLLSALLGENGDQISKDMANDFTQNELEFGGQRLKNTWDIPDLENKLL 150
   ||| |||||:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|
86 MSNLSSALLGENGDQISKDMANGFYKNELDFEGQYPQNIWNVPELENKPL 135
151 EDYSDDEDKLLALYFFASQELPMEANQQSNAANFFKVIDFLLILSAVTSLG 200
   ..|||:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|
136 SAYSDDDKLLALYFFSVQEI PLEENQQSNAARFFKLIDFLFTLSAVTSLG 185
201 KRIFSKNFYNGLETKSLENYIERKKLSKPFFRPPQKLPDGRGTGYLAGPTK 250
   :|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|
186 RRIFSKNFYNGLEAKSLENYIERKKLSKPFFRPPQRLPDGRIGYLAGPTE 235
251 APKLPTTSSTATTSTAASSNWRVSLQKLRDNPSRNTFMKMDDAAKRKYSS 300
   ||| |||:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|
236 APK.....WRVSFKELKNNKSRNGFSNMEGAACKQYSS 268
301 FIKEVQKGNDFRAAAASIGTKSGSNFEKLQGRDLYSIRLSQEHRTFSIN 350
   |||||:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|
269 FIKEVQKGNAPQTAASIGTASGSNLEKLPN.NLYSVRLSQKDRVTFTQN 317
351 NTDQIMEIQSVGTHYQNI 368
   :|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|:|
318 DTDNTMTVHHSVGYTHYKNI 335

```

FIGURE 7

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**Figure 8.**

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00562

A. CLASSIFICATION OF SUBJECT MATTER																						
Int Cl ⁶ : A01H 5/00; A01N 63/02; C07K 14/24; C12N1/11, 1/19, 1/21, 5/14, 7/01																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols)																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WEBANGISS: Sequence id's No. 3 and 4; MEDLINE: Keyword: Xenorhabdus STN file reg: sknfyngletkslenyiericklskpfirppqkdpdgrtylagpdkapklptststtaassnwr/aysdddkllalyffavqepieenqqsnaarffklidffilsavtslgrriifsknfyngleakslen/sqep																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
A	WO 95/00647 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 5 January 1995 See whole document especially Tables 1 and 2	1-19																				
<input type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex																						
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"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 20 August 1998		Date of mailing of the international search report 26 AUG 1998																				
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer DAVID GRIFFITHS Telephone No.: (02) 6283 2628																				

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00562

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	95/00647	AU	69916/94
		EP	705340

END OF ANNEX